Research Proposal for Odonate Fitness in Relation to Imidacloprid and Varying pH

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Abstract

Both neonicotinoids and varying pH levels affect odonate growth rates, which in turn can affect fitness. This proposal aims to assess how the combined stress of living in non-neutral water (pH = 5.0, 6.0, 8.0, and 9.0) and being exposed to a continuous, sublethal dose of 0.02 μg/L of the neonicotinoid imidacloprid will affect *Anax junius* stadia growth rates. Mortality rates will also be measured.

Terms and Definitions

Wing Sheaths
: Sometimes casually referred to as 'wing pads.' These are the instars' housings for what will eventually develop into wings during later stadia.

Instar
: Used to refer to an odonate in a certain life stag (e.g. the third instar).

Stadium (plural: stadia)
: The life stage(s) of odonates in between molts.

Fitness
: The ability to survive to a reproductive age, find a mate, and produce viable offspring.

Literature Review

With the widespread use of neonicotinoids and the prevalence of both acidic and alkaline waters, it has become increasingly important to monitor how environmental conditions affect aquatic macroinvertebrates.

Water pH

pH is important to aquatic insects such as *A. junius*. Most studies on aquatic insects use water at a neutral pH. However, many lakes, streams, and rivers are fairly acidic. Historically, this was due to the sulfuric compounds and other pollutants released from power plants, especially coal power plants. Approximately 6.9% of freshwater bodies in the Adirondack Mountains, 2.9% of freshwater bodies in New England, and 3.5% of freshwater bodies in the Mid-Atlantic were considered "chronically acidic" (a body of water with an acid neutralizing capability less than μeq/L) by the Environmental Protection Agency (EPA) (EPA, 2014). Ultimately, although surface waters that were formerly acidified from sulfuric oxides are recovering thanks to air pollution control

measures, many surface waters remain acidified for a number of reasons (Environmental Protection Agency [EPA], 2014).

As calls for metal mining increase, the risk of acid mine drainage increases as well. Acid mine drainage is a common environmental effect of mining activities in sulfur-rich ores. It has been well documented in numerous case studies, and approximately 10,000 miles of rivers and streams have been contaminated by acid mine drainage (Jennings et al., 2008; Copper et al., 2003). Mining exposes sulfur rich ore to light and water, which react to create sulfuric acid, acidifying the site. This acidic runoff in turn tends to degrade the ores around it, releasing harmful heavy metals such as nickel, lead, and cadmium (Johnson & Hallberg 2005). All of these heavy metals are linked to health problems such as chills, fever, muscle pain, lung damage, and kidney disease (OSHA, n.d.). Many of the studies on acid mine drainage reported pH as low 4.5 (Jennings et al., 2008).

Additionally, fracking can change hydrology in many ways, including pH. For an average well, 20 million liters of waters are forced into the gap along with 200,000 liters of acids, biocides, friction reducers, and surfactants (Howarth et al., 2011). Although pH is generally a secondary concern in the event of a fracking leak, pH is nonetheless an important aspect situation.

Finally, some streams and lakes may be naturally acidic. For instance, peatlands and some wetlands, especially those dominated by sphagnum species and conifers, can be naturally acidic (Minnesota Department of Natural Resources [MN DNR], n.d.).

Human interference is generally more likely to result in acidified waters, but it is still valuable to note that high alkalinity or basicness is also possible. This is often due to an underlying layer of rocks, such as limestone, or high levels of calcium in the soil, which leach into surrounding waters and increase alkalinity. Counterintuitively, acid rain can also cause increased alkalinity because acidic waters can dissolve rocks and impervious man-made surfaces, releasing alkaline minerals. Kaushal et al. (2013) found that in the eastern United States, almost 2/3 of sites studied had become more alkaline in a large part due to this acidic weathering.

Although it is widely acknowledged that pH impacts fitness of aquatic life forms, there is a dearth of studies on how it impacts odonates (Jennings et al., 2008; Copper et al., 2003). Bell (1971) found that the odonate species *Ophiogomphus rupinsulensis* and *Boyeria vinosa* died after 30 days' exposure of water with a pH of 4.30 and 4.42, respectively. The only study of pH impacts on odonate species *Anax junius* (Punzo, 1988) found that at a temperature of 20 °C, environmental pH of 3.0 reduced survivorship to 10%, while a pH of 7.0 produced over 95% survivorship (Punzo, 1988).

Overall, there is a gap in literature on acidification effects beyond mortality on odonate fitness and growth rates, and almost no studies seem to have been conducted on higher pH values (7.0 – 9.0). Studying both acidic and alkaline waters is important because both occur regularly in surface waters (MN DNR, "LakeFinder", n.d).

Neonicotinoids

Another pertinent stressor that many aquatic insects face today is neonicotinoid runoff. Numerous studies have shown that neonicotinoids, a class of insecticides, are increasingly present in rivers, lakes, and streams (Morrissey et al., 2015). Neonicotinoids

are licensed in over 120 countries and are valued at $2.6 billion (of which imidacloprid makes up 41%) (Goulson, 2013). Since some neonicotinoids are highly soluble, neonicotinoids applied to terrestrial systems frequently move to surface and ground waters. One literature review of neonicotinoids in water systems found that the chemicals were widespread in water systems across nine countries, with 89% of surface water samples in California, 27%-93% of water samples from Sydney, Australia, and 36%-91% of water samples in Canadian prairies testing positive for the presence of imidacloprid (Starner and Goh, 2012; Sanchez-Bayo, 2006; Main et al., 2014). Since neonicotinoids cause a variety of harmful effects on invertebrates—including paralysis, delayed development, disruption of cognitive functions, the destruction of key parts of the nervous system, reduced ability to learn and remember events, interrupted navigation abilities, increased susceptibility to pathogens, and death—their presence in water systems has caused concern (Pisa et al., 2014).

Invertebrates are keystone species to many aquatic ecosystems. In many cases, they make up the bulk food sources for animals such as trout. These animals in turn contribute to a recreational fishing industry that in the U.S. alone is estimated to be worth about $42 billion and involve 33.9 million users (U.S. Fish and Wildlife Service [U.S. FWS], 2006). Consequently, studying invertebrate responses to acute and chronic neonicotinoid presence is relevant on both ecological and economical levels (Morrissey et al., 2015).

Thanks to the increased application and awareness of neonicotinoids, a considerable number of studies have focused on neonicotinoid impacts on various insects. Most studies have used imidacloprid, a common neonicotinoid. Clonthianidin and thiamethoxam were also highly prevalent, but since imidacloprid dominates 41% of the neonicotinoid market, this study will focus on imidacloprid (Goulson, 2013). This study will base experimental methods on previous studies. Table 1 reviews the experimental descriptions, treatments, variables, items measured, and results for over a dozen studies on imidacloprid's effects on aquatic organisms (attached as separate sheet). As seen in Figure 1, amphipods and Diptera were the most widely studied species, but odonates were still studied in a few experiments (18% of 23 experiments). Table 2 focuses on the experimental descriptions, species, level, and results for studies on imidacloprid's effects on odonates in particular (attached as separate sheet).

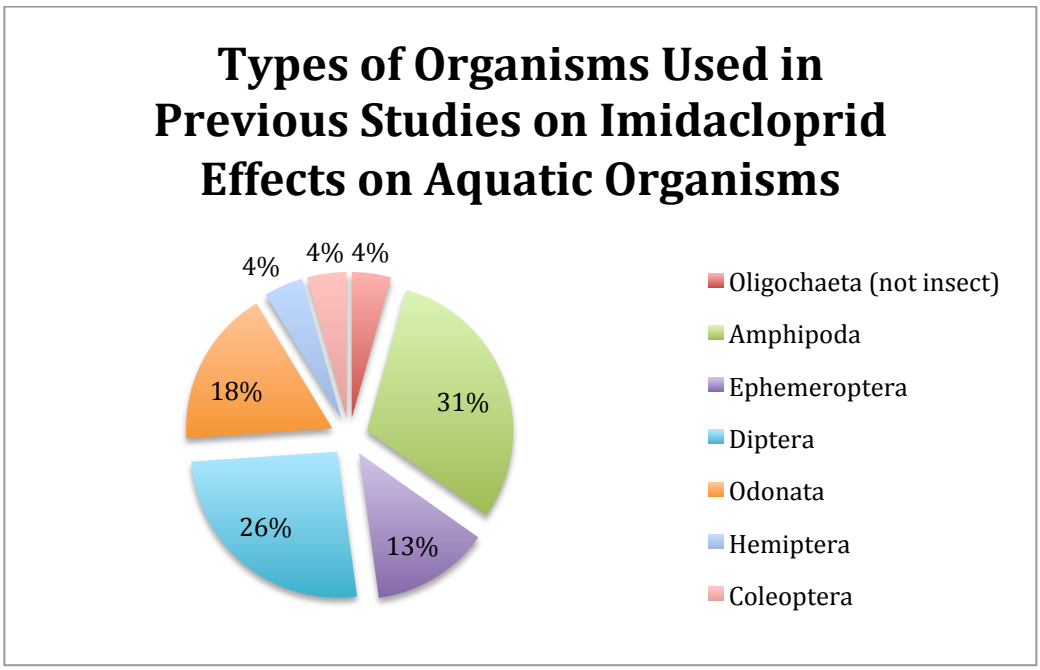

Figure 1: Species breakdown by order or class for literature review of 23 studies on imidacloprid's effects on aquatic organisms.

A common way to report effects of toxins on organisms is to note the level at which 50% of exposed organisms died (LC50). There was considerable variability in the studies reviewed, so those that did include LC50 information are summarized in Table 3.

Table 3: Review of LC50s by Order or Class

Order or Class	**Timing**	**LC50 (µg/L)**
Amphipoda	24-hr	146.4
Amphipoda	24-hr	185.6
Amphipoda	24-hr	155.8
Diptera	48-hr	19.9
Diptera	96-hr	5.81
Diptera	14 days	1.52
Diptera	96-hr	5.4
Diptera	96-hr	2.65
Diptera	48-hr	8.15
Diptera	28 day test	0.91
Diptera	28 day test	11.46
Ephemeroptera	24-hr	2.1
Ephemeroptera	96-hr	0.65

pH and neonicotinoids

The physical properties of neonicotinoids change in response to pH. Some neonicotinoids degrade more quickly in acidic conditions, while others may potentially degrade more quickly in alkaline conditions, but stay stable at acidic and neutral pHs (Bomatin, 2015).

Regardless of pH level, imidacloprid disintegrates in water through photolysis and hydrolysis. Sunlight is key to this degradation. Aregahegn et al. (2017) found that with adequate ultra-violet light, a solid film on a neonicotinoid can degrade within hours, yet in the dark it can persist for months. The chemical can degrade into up to nine metabolites, including "a cyclic guanidine derivative, a cyclic urea, an olefinic cyclic guanidine, and two fused ring products" (Morrissey, 2015 p. 297). Imidacloprid's most important metabolite is1-[6-chloro-3-pyridinyl) methyl]-2-imidazolidone (IMD-UR). Under 24-h to 48-hour exposure bursts, these metabolites appear to be less toxic than the parent compound (imidacloprid) (Morrissey, 2015). However, they can still have effects. For example, Morrissey (2015) reported that *Gammarus fossarum* react negatively to 6-chloronicotinic acid. Their behavior became erratic, and antioxidant enzyme activity faltered.

On top of its normal degradation in water over time, imidacloprid can further react in acidic and basic conditions. A literature review on neonicotinoid properties noted that five out of six studies found that imidacloprid was fairly stable at a neutral pH of 7.0, but degradation increased as pH increased to about 9.0 (the sixth study found the opposite result) (Bonmatin et al., 2015). Considering the low number of studies and contradictions, there are many opportunities for future research on the effects of neonicotinoids in acidic and basic waters. A study analyzing both would give policymakers and citizens a better idea of how these combined stressors affect odonates.

I propose to test how the simultaneous stress of unusual pH conditions and neonicotinoids may affect the aquatic insect *Anax junius*. To do this, I will conduct separate trials of pH and neonicotinoids in three phases:

1. Assessment of how varying pH levels impact *A. junius* growth and survival
2. Assessment of how chronic exposure to imidacloprid (a neonicotinoid) at 0.02 µg/L impacts *A. junius* growth and survival
3. Assessment of how varying pH levels with a background chronic exposure to imidacloprid impacts *A. junius* growth and survival

The control for phase 1 and 2 shall be raising nymphs in water with a pH of 7.0 and no imidacloprid. The control for phase 3 will be the results from phases 1 and 2.

For the first phase, I propose to study pH effects on growth rates in waters with a pH of 5.0, 6.0, 8.0, and 9.0. This range was selected to cover both acidic and basic waters without stressing the larvae enough that they will die. Punzo (1988) found that water with pH much below 5.0 studies resulted in extremely high mortality. I do not want mortality to rise above 50% because it will reduce the amount of data I can collect on growth rates.

For the second phase, a wide range exists for potential dosages of imidacloprid. I propose exposing the nymphs to 0.02 µg/L because the literature review revealed that chronic exposure at 0.012 – 0.035 µg/L should be enough to result in a response but moderate enough to avoid immediate mortality (Van Dijk et al., 2013 and Morrissey et al., 2015). By contrast, odonates and other aquatic insects showed LC50 levels of about 0.2-0.65 µg/L (Morrissey et al., 2015 and Goulson, 2013).

Finally, the third part of the study will involve rearing *A. junius* in waters with a pH of 5.0, 6.0, 8.0, and 9.0 while exposing them to 0.02 µg/L of imidacloprid.

I selected the common green darner, *Anax junius* because is prevalent, practical, and important. *A. junius* is present in freshwater ecosystems across all 50 states in the U.S.A., the West Indies, Guatemala, Belize, Costa Rica, and England, making research especially relevant to all of those locations as well as easily replicable (Abbott, 2017). It plays an important role in freshwater ecosystems as both prey to fish and predator of mosquitoes. *A. junius* is also easy and inexpensive to catch and rear.

As a dragonfly, *A. junius* is also important to the public. The National Park Service is already using dragonflies for large-scale citizen-led surveys because of their large size, charisma, and clearly identifiable features (National Park Service [NPS], 2016). Finally, it is useful to know how pollution may affect dragonflies because they are a charismatic "flagship species" for maintaining biodiversity. People like dragonflies for their own sake, and by gathering information to protect dragonflies people can help conserve entire aquatic habitats that benefit many species. For instance, Lemelin (2007) found that dragonfly educational outings, counts, and events have been steadily increasing. Butterfly tourism already has significant traction – the Monarch Butterfly Biosphere Reserve in Mexico already gets 250,000 people per season – and many hope that dragonflies can be similar flagship species for aquatic ecosystems (Lemelin, 2007). Overall, *A. junius'* widespread populations, importance as a predator, convenience as a lab subject, and charisma as a potential flagship species make it an ideal choice for research on anthropogenic impacts on aquatic ecosystems.

There are currently very few studies on how both neonicotinoids *and* different pH levels may affect the fitness of the aquatic insect *A. junius*, yet the benefit of knowing how different factors in the environment may work together are considerable (Morrissey et al., 2015). Knowing these relationships can help equip citizens, policy makers, and non-governmental organizations (NGOs) with knowledge on how to help both dragonflies and the ecosystems they rely upon.

Proposed Methods

Sample Size: I propose collecting 12 nymphs as a control (no pH nor neonicotinoids), three nymphs for each of the four pH levels of the pH only test (three nymphs for pH = 5.0, three nymphs for pH = 5.0, three nymphs for pH = 8.0, three nymphs for pH = 9.0), twelve nymphs for the neonicotinoid only test, and twelve nymphs for the combined pH and neonicotinoid test (three nymphs for each pH level, and all nymphs will also be exposed to 0.02 µg/L of imidacloprid). This number is based on previous work by Thompson, the maximum number of nymphs recommended by the National Park Service

odonate citizen science program (NPS, 2016), and recommendations from advisors Ami Thompson and Karen Oberhauser.

Collecting odonates

I will collect *A. junius* from ponds within Crow Hassan Park Reserve. This site was selected because it has been used for both previous odonate studies and mosquito studies. The Minnesota Pollution Control Agency uses ponds at Crow Hassan Park Reserve when comparing insecticide contamination because there is no spraying for mosquito control on or near the reserve (Thompson, n.d.). I will use aquatic nets (38 x 23 cm wide with 0.5 cm mesh) to catch nymphs. For maximum collection yield, I will drag the nets along the vegetation at about three feet deep. *A. junius* will be identified by eye, placed in plastic containers with pond water and some native vegetation to hold onto, and transported back to the lab in insulated foam coolers.

Control Trial

I will place individual *A. junius* in plastic containers full of dechlorinated water with a few rocks and other substrate. Light and temperature affect odonate growth, so these will be controlled. Trottier (1971) found that a minimum of 8.7° ± 0.1 °C and about 1332 degree-days ± 1% were needed to develop odonates over the summer, and 20.5% more degree-days were needed for winter development. To emulate ideal summer conditions, 60 Watt light bulbs will be kept on for 16 hours each day above the containers, and the nymphs will be raised at 28°C ± 3.0 °C (Thompson, n.d.). I will feed nymphs ~1/8 teaspoon of aquatic blackworms (*Lumbriculus Variegatus)* every day. Based on Thompson's previous work, this is more than *Anax junius* usually eats in one day, and will thus ensure that food is not limiting. I will also empty the nymph containers, clean the containers with a pipette, and replace water with fresh dechlorinated water each day.

pH Trial

The following methods are based on Punzo (1988). I will rear twelve odonates in with water of varying pH levels (pH 5.0, 6.0, 8.0, and 9.0). I will create a solution with pH = 5.0 by adding (Endo Glassware Graduated pipette, 0.5/0.005 mL) 0.05 mL of hydrochloric acid (The Lab Depot, Inc. Hydrochloric Acid, 1.0 N [1.0 M], CAS No 7647-01-0) to 5,000 mL of dechlorinated water. I will create a solution of pH = 6.0 using the same process, except with 0.005 mL of HCl instead of 0.05 mL.

I will create a solution with pH = 8.0 by adding (Endo Glassware Graduated pipette, 0.5/0.005 mL) 0.005 mL of sodium hydroxide (Sigma-Aldrich Sodium hydroxide solution, volumetric, 1.0 M, ID = S2567) to 5,000 mL of dechlorinated water. I will create a solution of pH = 9.0 using the same process, except with 0.05 mL of NaOH instead of 0.05 mL.

I will store the 1.0 M HCl, the 1.0 M NaOH, and the 5,000 mL of stock solutions in closed, clearly labeled containers in a well-ventilated room away direct sunlight (as according to the Safety Data Sheets). Every day, I will give the nymphs fresh water of the appropriate acidity, and I will test pH with a pH meter and probe (Oakton Handheld Meter with ATC, #UX-35613-50, and Oakton Single Junction pH electrode, #UX-59001-65).

Neonicotinoid Trial

The following methods are based on Scholer and Krischik (2014). I will create a 100,000 µg/L stock solution by adding 0.02 grams (Sartorius ED323-CW milligram balance) of analytical grade imidacloprid (37894 Sigma-Aldrich Imidacloprid PESTANAL® analytical standard, Milwaukee, WI, CAS Number: 138261-41-3, 100 percent) to 200 mL of dechlorinated water. I will stir this to ensure the imidacloprid dissolves (Thermo Scientific™ Komet Stir Bar). I will create a dilution of 0.02 µg/L by pipetting (MicroPette™ Single Channel Variable, Adjustable 0.1-2.5 µL Pipettor) 1.0 µL of the stock solution into 500 mL of dechlorinated water. I will store this dilution at 5.5°C in bottles with red glass (PYREX Low Actinic 1 L Round Media Storage Bottles) to reduce light exposure because imidacloprid degrades in sunlight. I will make the stock solution every 3 weeks and the dilution weekly.

I will fill each nymph's container with the 0.02 µg/L dilution. I will also keep a positive control container exposed to the same light cycle as the nymphs but without a nymph. This positive control is important because nymphs breathe, exhale, eat, and defecate, and these functions may change the chemistry of the water. Each time I make a new stock solution, I will collect 20 mL of the 100,000 µg/L stock solution, 20 mL of the 0.02 µg/L dilution from the positive control, and 20 mL of the 0.02 µg/L dilution from one of the *A. junius* containers. The stock solution, positive control dilution, and the used dilutions will all be stored at 80 °C until the end of the experiment (they can be stored for up to 1 year), at which point they will be shipped on dry ice to USDA, AMS, Gastonia, NC. This is an EPA-certified laboratory. It will use a standard USDA method to analyze the residue for imidacloprid parent compounds and metabolites.

Combined pH and neonicotinoid trial

I will follow the same methods outlined in the previous two trials. 12 nymphs shall be raised in waters of non-neutral pH with a background chronic exposure to 0.02 µg/L of imidacloprid.

Measuring growth rate and mortality

Growth rate and mortality were selected because they can indicate fitness. Odonate nymphs that are able to grow larger than others of the same kind tend to be able to catch more prey and compete more effectively for mates. Early mortality results in zero ability to pass on genetic code.

Odonates go through an incomplete metamorphosis as nymphs or naiads (Tennessen, 2008). As nymphs, odonates go through 10-15 stadia/instar (Tennessen, 2008). I will measure head width and mortality according to Table 4. The change in size will allow researchers to consistently determine nymph growth rate (body length is not a consistent indicator of size because nymphs can elongate or scrunch their abdomens).

Table 4: Measurements Guide

Fitness Indicators	**Methods and background**
Head width	I will use an ocular micrometer to measure the head width in millimeters.
Mortality	I will note mortality rates

Fieldwork Experience

Although I was not able to complete the data collection for this proposed experiment, I have previously assisted Ami Thompson and fellow Monarch Lab members with odonate research and field surveys. We captured dozens of *A. junius* at Crow Hassan Park Reserve. Some individuals were taken back to the Monarch Lab, where I assisted in rearing them. Thanks to previous work by Thompson and members of the Dragonfly Society of the Americas, we found that it was easiest to rear dragonflies in small plastic containers (DuBois and Tennessen, 2016). They were primarily fed aquatic blackworms (*Lumbriculus Variegatus)*, but when the worms became scarce we found that they would also eat other macroinvertebrates including snails, leeches, caddisfly larvae, and amphipods. This rearing method is almost identical to the proposed methods for capturing larvae and rearing the control treatment (DuBois and Tennessen, 2016).

I have previously created acidic waters in Chemistry I and II laboratories, and I have experience measuring pH, DO, temperature, and alkalinity from previous coursework.

Expected Results

Expected Control Results

Ami Thompson has been rearing *A. junius* in the lab for over a year and has observed a mortality and emergence rate described in Table 5 (Thompson, n.d.). Based on Thompson's preliminary results, I expect a background mortality of approximately 13%.

Table 5: Thompson Control Results

	# Nymphs
Successfully emerged	29

Still Nymphs	10
Died	6
Total	45
Total % of dead/alive	6/45 = 13%

Figure 2 expected head width increase over time for control nymphs.

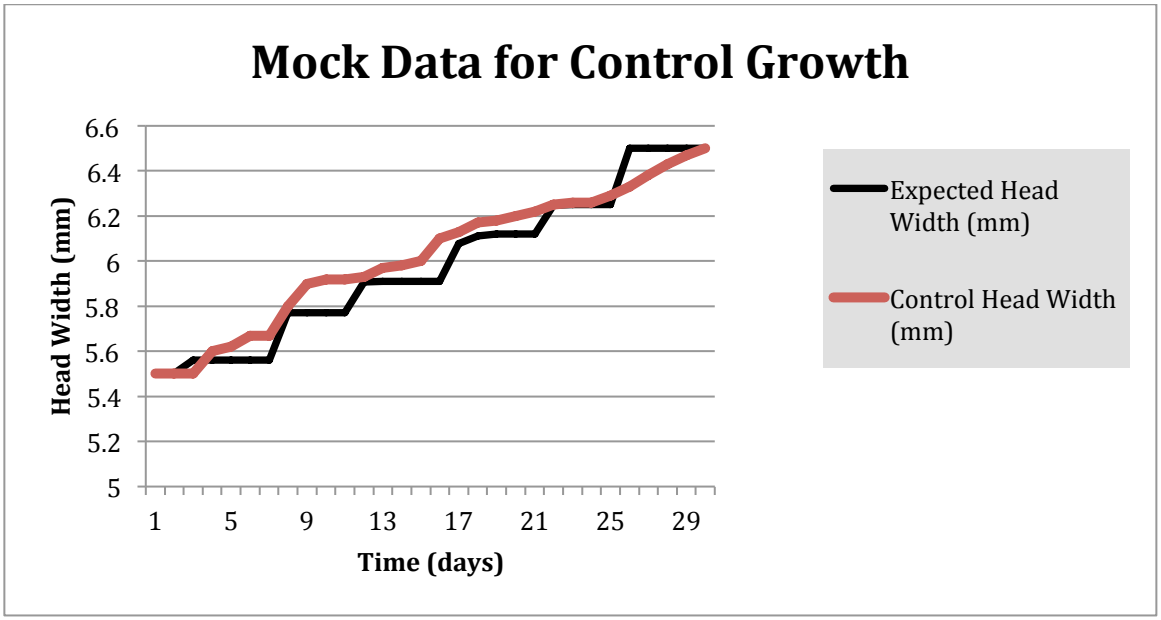

Figure 2: Mock graph for control growth. The black line shows the expected linear growth; the red line shows an example of how actual data may follow a stair-step-like pattern. This pattern occurs because head width tends to increase after the subject goes through each ecdysis, and then stay about constant until the next ecdysis.

Expected pH Results

Mortality

I expect that odonate mortality will increase when the pH is higher or lower than 7.0.

Null Hypothesis: $u_0 = u_{pH}$
Hypothesis: $u_0 \neq u_{pH}$

Where u_0 is the percentage of odonate mortality for the control and u_{pH} is the percentage of odonate mortality for the pH trial.

Figure 3 shows a scenario where I fail to reject my null hypothesis in the upper bars and successfully reject my null hypothesis in the lower bars.

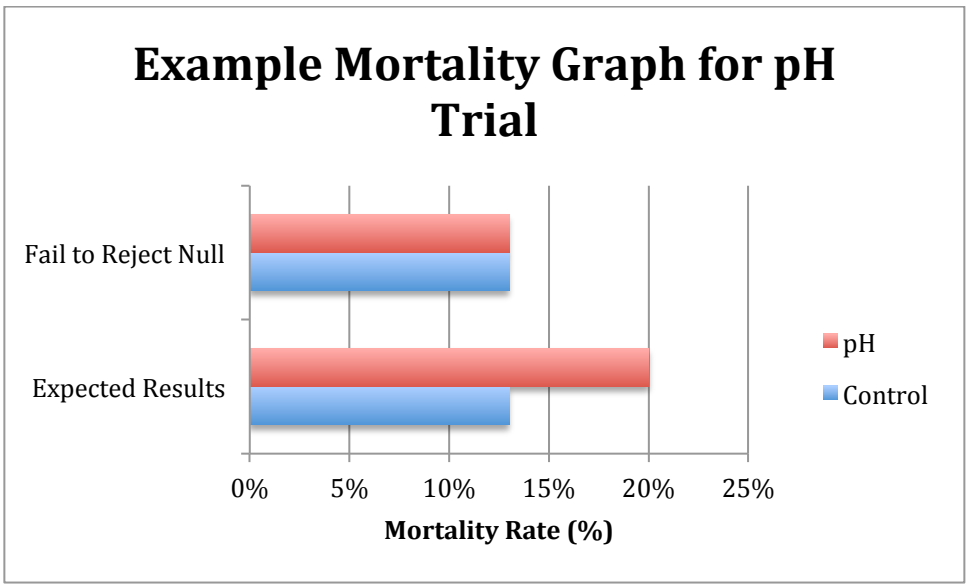

Figure 3: Example mortality graph for pH trial

Growth rate

As seen in Figure 4, I expect that odonate growth will be fastest at pH of 7.0 and decrease as pH gets higher or lower than 7.0. Figure 5 shows what the trend might look like if I failed to reject the null hypothesis.

Null Hypothesis: $g_0 = g_{pH}$
Hypothesis: $g_0 \neq g_{pH}$

Where g_0 is the growth rate for the control (the 12 nymphs raised in a pH of 7.0) and g_{pH} is the growth rates of the nymphs raised at pH of 5.0, 6.0, 8.0, and 9.0.

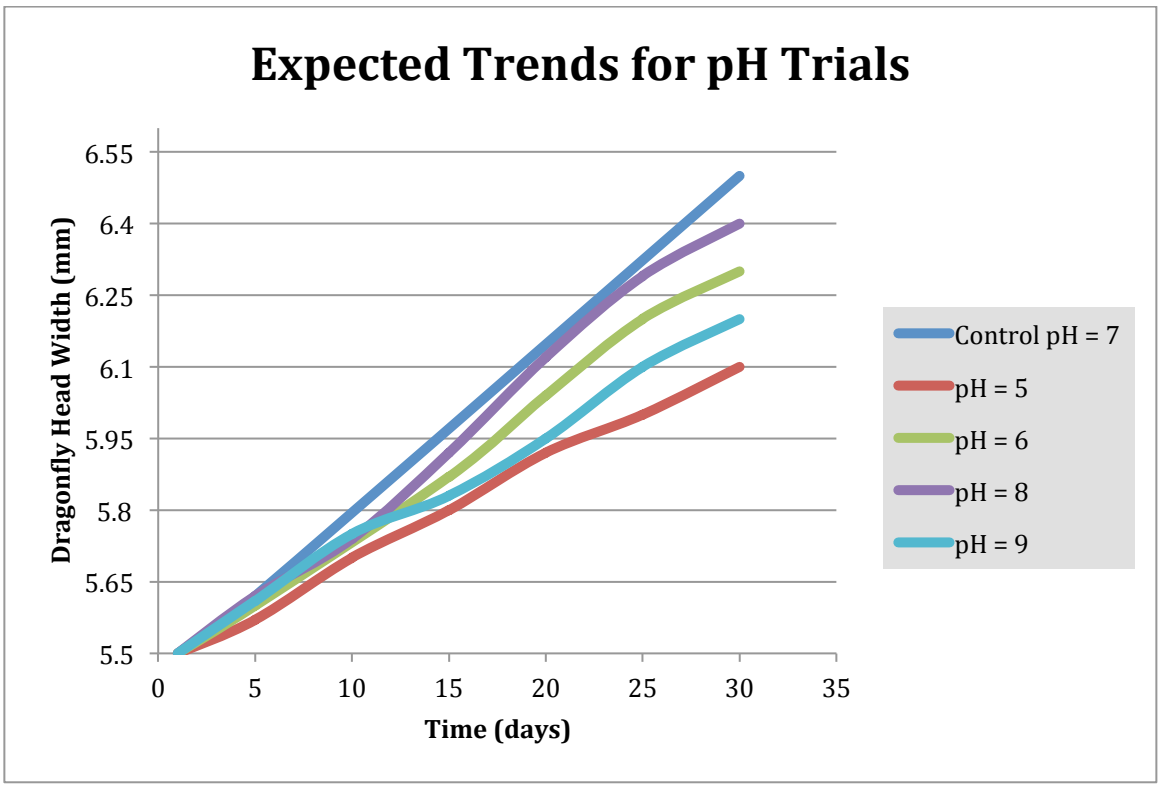

Figure 4: Expected linear trends for pH trials.

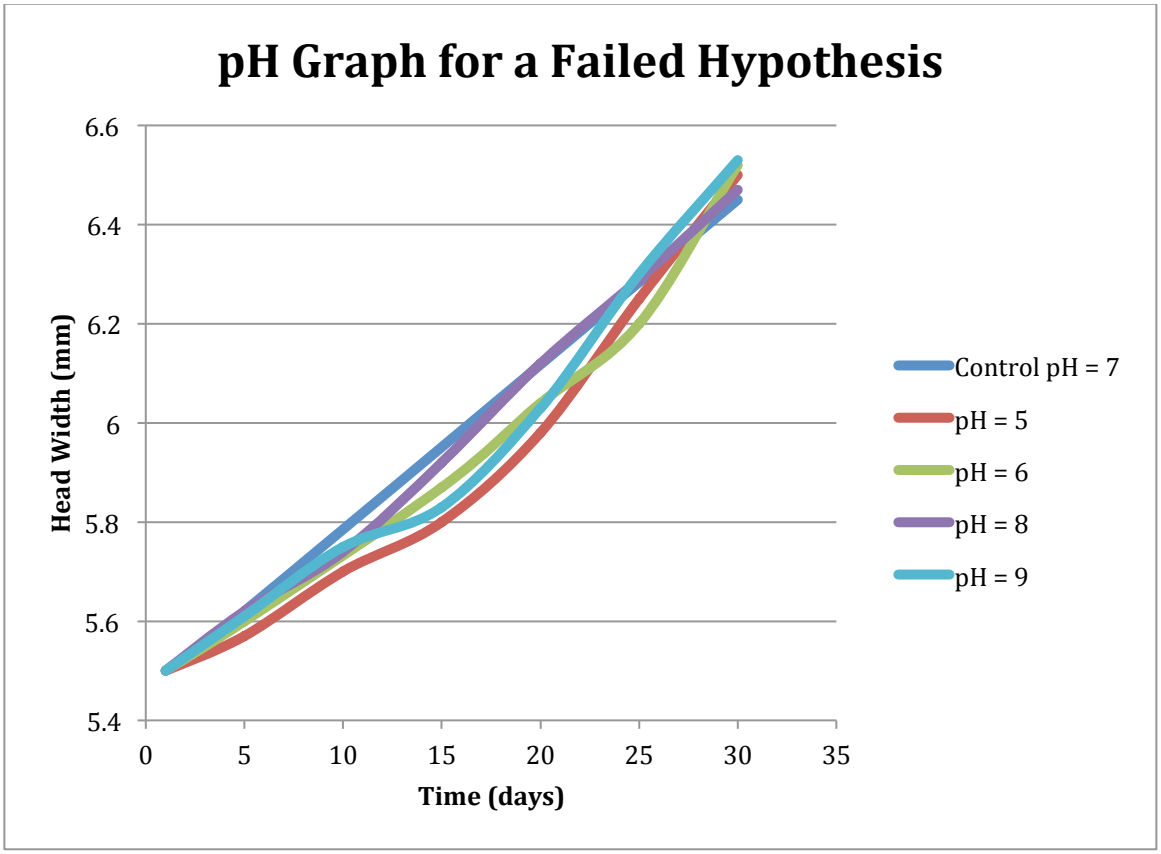

Figure 5: pH Graph for a Failure to Reject the Null Hypothesis

Expected Neonicotinoid Results

Mortality

I expect that odonate mortality will increase when nymphs are raised in neonicotinoid-spiked waters.

Null Hypothesis: $u_0 = u_n$
Hypothesis: $u_0 \neq u_n$

Where u_0 is the percentage of odonate mortality for the control and u_n is the percentage of odonate mortality for the pH trial.

Figure 6 shows a scenario where I fail to reject my null hypothesis in the upper bars and successfully reject my null hypothesis in the lower bars.

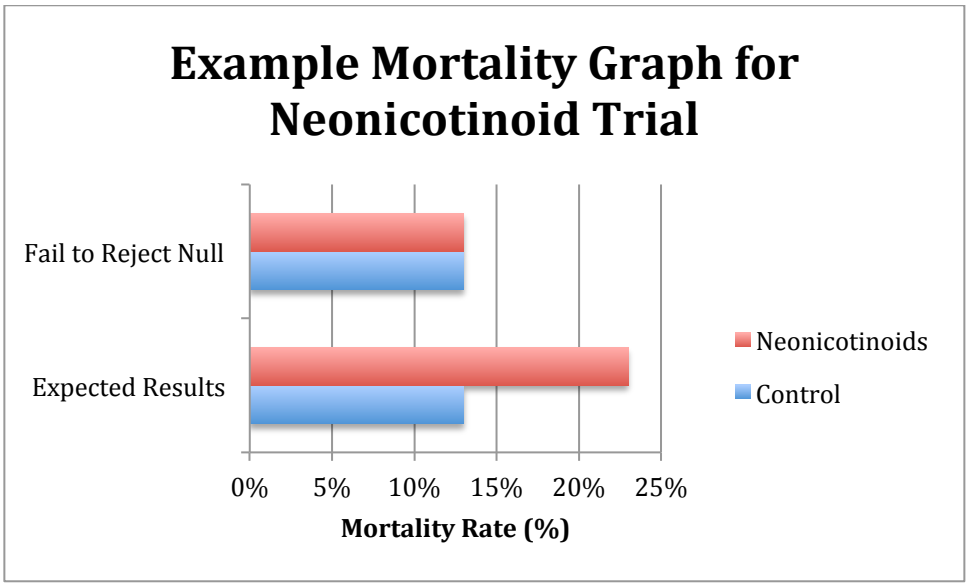

Figure 6: Example mortality graph for neonicotinoid trial

Growth rate

As seen in Figure 7, I expect that the control odonate growth will be faster than the neonicotinoid trial. Figure 8 shows what the trend might look like if I failed to reject the null hypothesis.

Null Hypothesis: $g_0 = g_n$
Hypothesis: $g_0 \neq g_n$

Where g_0 is the growth rate for the control and g_n is the growth rate of the neonicotinoid trial.

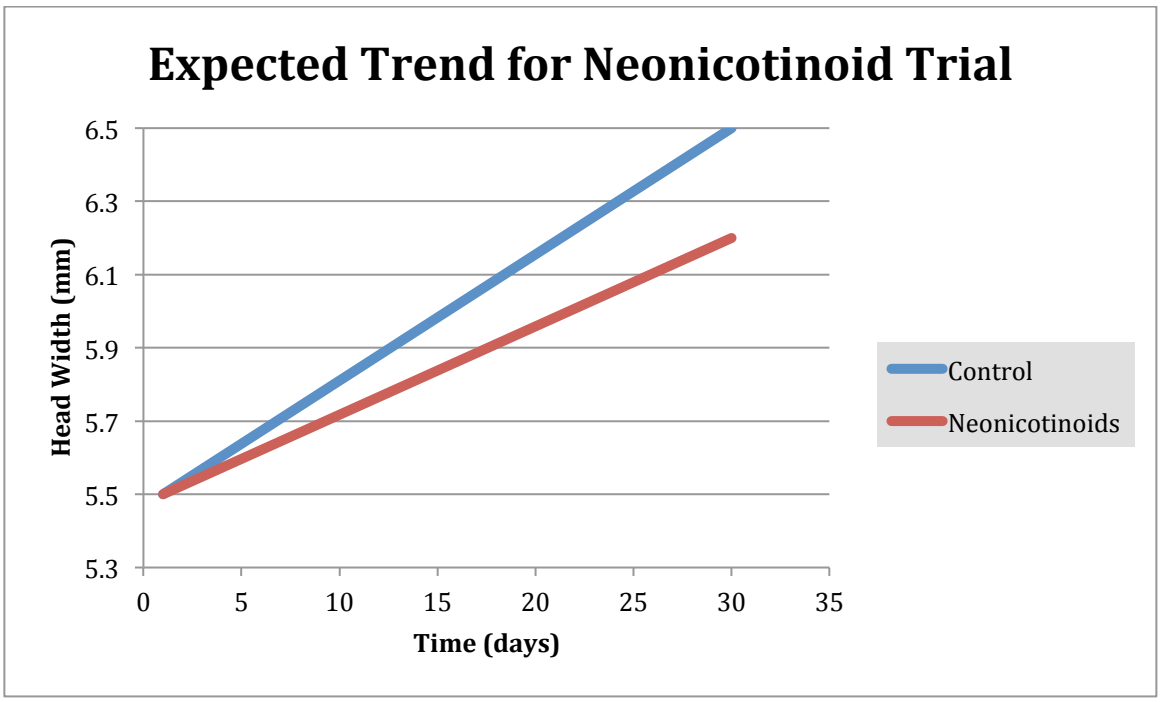

Figure 7: Expected trend for neonicotinoid trial, where I successfully reject the null hypothesis

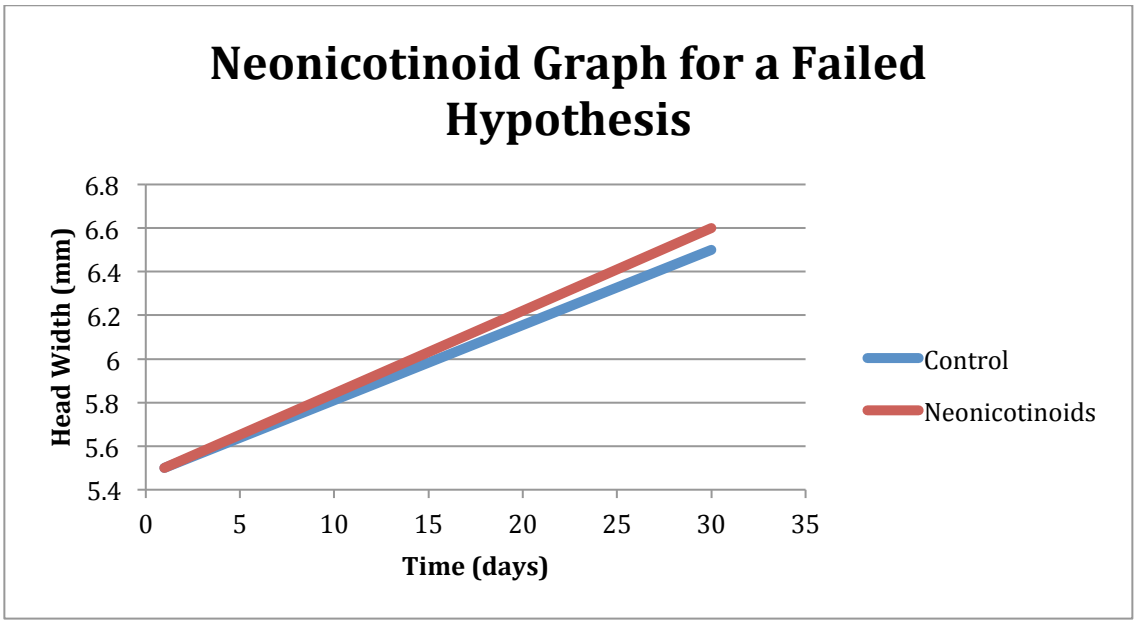

Figure 8: Expected trend for neonicotinoid trial, where I fail to reject the null hypothesis

Expected combined pH and Neonicotinoid results

Mortality

I expect that odonate mortality in the combined neonicotinoid and non-neutral pH trial will be higher than the neonicotinoid-only trial and the pH-only trial.

Null Hypothesis: $u_{pH} = u_c$ and $u_n = u_c$
Hypothesis: $u_{pH} \neq u_c$ and $u_n \neq u_c$

Where u_{pH} is the percentage of odonate mortality for the pH-only trial, u_n is the percentage of odonate mortality for the neonicotinoid-only trial, and u_c is the percentage of odonate mortality for the combined neonicotinoid and pH trial.

Figure 9 shows a scenario where I fail to reject my null hypothesis in the upper bars and successfully reject my null hypothesis in the lower bars.

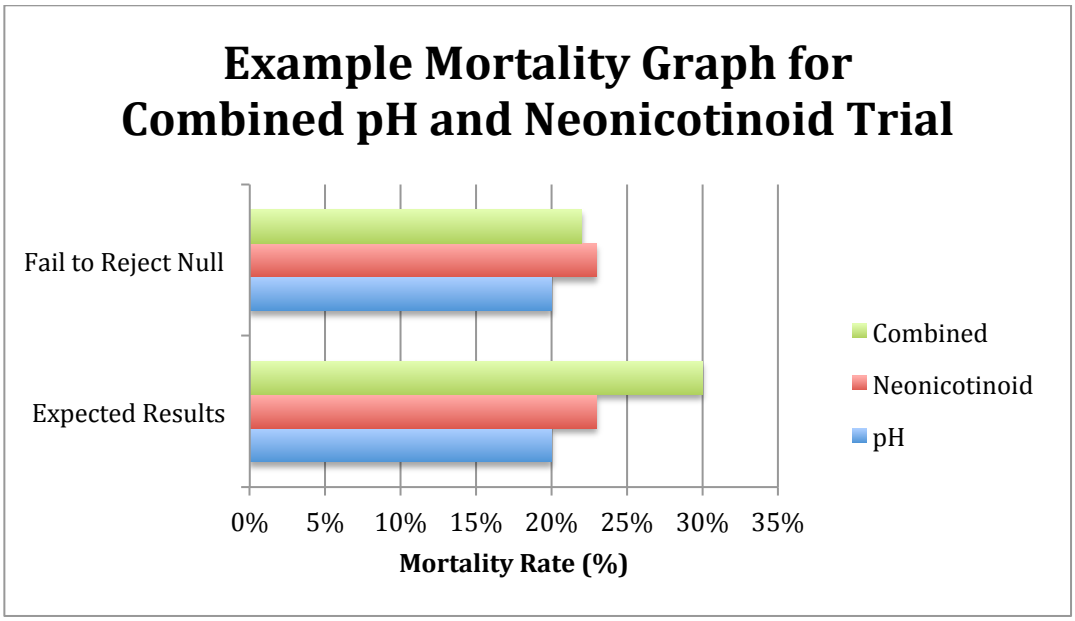

Figure 9: Example mortality graph for combined pH and neonicotinoid trial

Growth rate

As seen in Figure 10, I expect that odonate growth will be slowest in the combined pH and neonicotinoid trial, moderate in the neonicotinoid-only and pH-only trials, and fastest in the control trial. Figure 11 shows what the trend might look like if I failed to reject the null hypothesis.

Null Hypothesis: $g_{pH} = g_c$ and $g_n = g_c$
Hypothesis: $g_{pH} \neq g_c$ and $g_n \neq g_c$

Where g_{pH} is the growth rate for the pH-only trial, g_n is the growth rate for the neonicotinoid-only trial, and g_c is the growth rate for the combined neonicotinoid and pH trial.

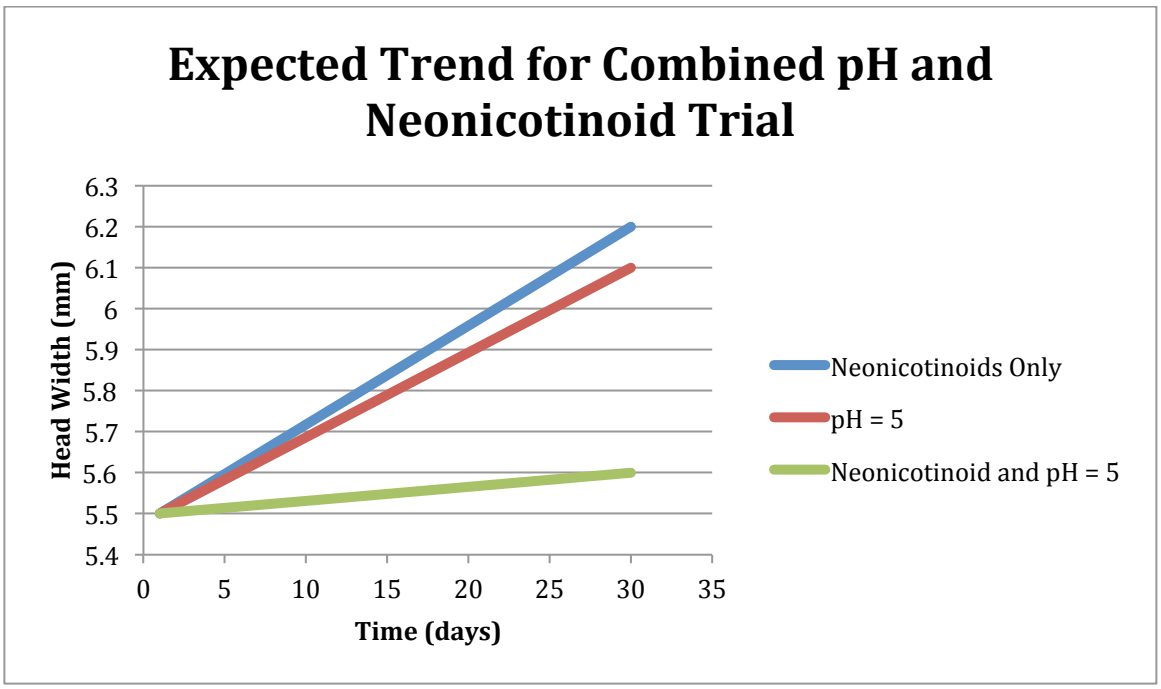

Figure 10: Mock graph of expected trend for the combined pH and neonicotinoid trial for pH=5.

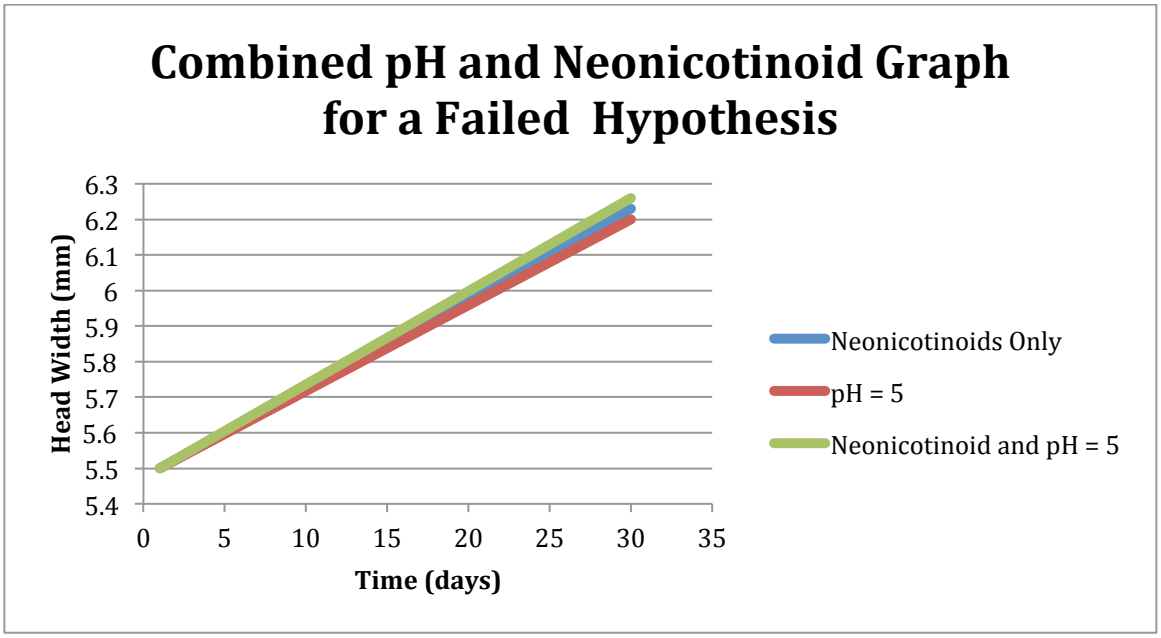

Figure 11 shows what the trend might look like if I failed to reject the null hypothesis.

Discussion

I expect that raising *A. junius* in non-neutral waters will result in a higher mortality rate and a lower growth rate. This is because the literature suggests that extremes are stressful for aquatic insects (Punzo, 1988; Jennings et al., 2008; Copper et

al., 2003). It would also be interesting to note whether acidic waters are more stressful to *A. junius* as compared to alkaline waters because very few pH studies have considered alkaline waters. Overall, successful rejection of the null hypothesis would both support previous literature and emphasize the importance of acid mine drainage, acid rain, and pollution on aquatic ecosystems. By contrast, a failure tor eject the null hypothesis would suggest that either *A. junius* are unusually hardy, or the methodology was flawed.

Similarly, I expect that raising *A. junius* in neonicotinoid-spiked waters will result in a higher mortality rate and a lower growth rate. Almost all of the literature on neonicotinoid impacts on aquatic organisms (Table1) found that imidacloprid increased mortality. Only one previous study, Chang et al. (2007), was unable to link increased mortality with imidacloprid presence. This was likely because Chang et al. used extremely low doses. Most literature in Table 1 also found that sublethal imidacloprid doses negatively affect other aspects, including feeding rates, gestation, mobility, symmetry, lipid content, and growth, (Agatz et al., 2013; Alexander et al., 2007; Camp et al., 2016; Chang et al., 2007; Nyman et al., 2013; Stoughton et al., 2008). Consequently, I conclude that successful rejection of the null hypothesis would support prior literature and strengthen the case for careful monitoring of how runoff from insecticide-dosed areas may affect surface waters. On the other hand, failure to reject the null hypothesis would likely mean that higher doses are needed.

Finally, I expect that the combined trial of neonicotinoids and extreme pH levels will result in even higher mortalities and lower growth rates than either of the factors by itself. A successful rejection of the null hypothesis would be important because it would show that areas exposed to both stressors (i.e. any surface water that is exposed to both farm or garden runoff and either acid rain, acid mine drainage, fracking runoff, or other pollution) are especially vulnerable. This could help policymakers and landowners increase protections for vulnerable surface waters.

One especially interesting trend to notice would be whether the impacts are additive (the negative stressors add together) or synergistic (the sum of negative impacts is greater than any single stressor). Synergistic impacts would be important because they would show that the combined stressors could have severe impacts on aquatic ecosystems. In addition, they might indicate that the two stressors could interact in unpredictable ways, making it more difficult to anticipate future problems to water systems.

In general, Thompson's team expected that growth would increase in a stairstep pattern along with each ecdysis, and head width size would increase with most molts. In this proposal I am simplifying this to linear growth; however, it is possible that neonicotinoids will cause growth to take on non-linear rates. For instance, the nymphs' food, aquatic blackworms (*Lumbriculus Variegatus),* has highly permeable skin and will likely take in neonicotinoid traces. Eating neonicotinoid-filled blackworms as well as being exposed to the insecticide over long periods of time might cause the exposed nymphs' growth rate to slow down over time. These trends could have implications to wider research on bioaccumulation of insecticides throughout the food chain.

A final trend to keep track of is whether growth rates in acidic waters are lower or higher than growth rates in alkaline waters. Imidacloprid disintegrates at different rates depending on acidity, and Morrissey et al. (2015) found that the metabolites are generally less toxic than imidacloprid. Most, though not all, studies also reported that imidacloprid

is stable at a neutral and acidic pH, but degrades more quickly in alkaline waters (Bonmatin et al., 2015). Consequently, it is possible that the nymphs in the alkaline neonicotinoid trials will die less often and grow more quickly than nymphs in more acidic neonicotinoid trials. Since different conditions can result in either more acidic or more alkaline waters, being able to link effects with acidity would be valuable when evaluating anthropogenic impacts on aquatic ecosystems.

Failure to reject the null hypothesis may suggest that odonates are hardier than expected. This could be positive for odonate populations. However, it would also mean that more research should be done on sublethal effects of neonicotinoids on odonates. Since odonates are also predators in aquatic ecosystems, it would be valuable to test whether combined pH and neonicotinoids have similar effects on prey species.

Conclusion

Ultimately, this study could be valuable for policymakers, concerned citizens, and the scientific community. Successful rejection of the control trials could help guide future choices when water systems are exposed to both pH extremes and imidacloprid. Additive effects would still be important, while synergistic effects would be more concerning because the interactions between the two stressors could be severe across invertebrate populations. Meanwhile, the relative rates of growth observed in the experiment would add to knowledge of the bioaccumulation of insecticides in subjects' bodies over time, while the disintegration of imidacloprid at different pH levels could expand knowledge of how the insecticide's metabolites affect aquatic organisms. Even a failure to reject the null hypotheses could still help lead to increased knowledge of where future studies could focus.

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Table 1: Review of Studies on Imidacloprid Effects on Aquatic Organisms

#	Experiment Description	Treatments	Variables	Measured	Results
1	Exposed *Gammarus pulex* to imidacloprid for four days. Gave them two days to acclimate to laboratory conditions, four days exposed to imidacloprid, and three days to recover. pH ranged from 7.4 - 7.9, O2 was always over 75%. Temp between 12.2 - 14.0 C. Optimum for that organism for pH was 7.2 - 7.8.	Five test concentrations of imidacloprid of 0.81 µg/L, 2.7 µg/L, 9.0 µg/L, and 30 µg/L, 100 µg/L. Used 99% analytical standard 99% purity PESTANAL Sigma Aldrich. Samples of stock solution taken at beginning and end of exposure phase, frozen at -22 C until preparation using liquid chromatography HPLC.	Imidacloprid concentration	Body mass, feeding rate (calculated by dividing amount of food eaten by the body mass of the individual.	2 way ANOVA on relative feeding rates. Graphed concentration (µg/L) against relative feeding rate (% of control); recovering creatures ate the most; exposed creatures ate the least.
2	Exposed mayfly *Epeorus longimanus* Eaton and oligochaete *Lumbriculus variegatus* to both 24-hour pulses of imidacloprid and four-day continuous exposure.	6 concentrations of 24-hr pulses of imidacloprid with concentrations of 0, 0.1, 0.5, 1.5, and 10 µg/L.	Imidacloprid presence; species	Mortality, algal biomass consumed	LC50 for 24-hr median in mayflies was 2.1 +/- 0.8 µg/L, larger mayfly instars (24-h LC50, 2.1 ± 0.5 µg/L; 96-h LC50, 0.65 ± 0.15 µg/L). Short 24-hr pulse of imidacloprid > 1 µg/L caused feeding inhibition. Oligohcaetes OK in 24-hour pulse, but 4 day continuous exposure reduced survivorship, feeding, gestation of both insects at concentrations between 0.5 to 10 µg/L .
3	Studied acute toxicity of	Used Malathion, Aldicarb,	Imidacloprid	Mortality	With neonicotinoids, 75% of *G.*

	different modes of action on amphipod *Gammarus pulex* vs. crustacean *Daphnia magna.* Used acute toxicity using 48-h and 96-h LC50s for *G. pulex* and 48-h EC50s for *D. magna.* Counted live/dead organisms after 24, 48, 72, and 96 hours by prodding organisms.	dichloroaniline, 2,4-dichlorophenol, 1,2,3-trichlorobenzene, 4,6-dinitro-o-cresol, 2,4,5-trichlorophenol, Ethylacrylate, 4-nitrobenzyl-chloride, Sea-nine, and Imidacloprid.	species		one order of magnitude for D. magna. *G. pulax* results for imidacloprid: LC50 at 404 – 512 nmol/L (146.4 µg/L – 185.6 µg/L) for 24-96 hours *D. magna* results for imidacloprid: LC50 was 430 nmol/L (155.8 µg/L)
4	Exposed *Chironomus riparius* to imidacloprid in pulses and through continuous exposure. Used *C. riparius* midges at 20 C in 16:8 light dark cycle. Exposed them to imidacloprid. Used twenty-five 7-day old larvae divided by 5 replicates with beakers containing 150 mL of pesticide solutions. For continuous test, exposed them for 10 days of exposure to a gradient of pesticide concentrations. For acute exposure, exposed for 4 days	Confidor 200 SL from Bayer CropScience, stored at 4C and protected from light. For continuous exposure, used 0.5, 1.5, and 4.5 µg of IMI/L after they earlier found that 48 h LC 50 is 19.9 µg/L (14.64 - 27.16 µg/L). For acute toxicity test, used concentrations of 1.25, 2.50, 5.00, 10.00, 20.00, 30.00, and 40.00 µg/L.	Imidacloprid presence, acute vs. continuous	Mortality and mobility. Measured movements using online Freshwater Biomonitor, to translate movements into frequency graphs.	*C. riparius* growth decreased by 34% -43% as imidacloprid increased after 96 and 240 h of exposure. When exposed for four days and transferred to clean medium, larvae showed no differences with growth.

	followed by 6 days of clean water. For the continuous test, every 48 hours; most of the test solution was renewed and animals were fed. Animals were not fed for acute test.				
5	Used low doses of imidacloprid in pulses against amphipod *Gammarus roeseli* in streams for 70 days. Studied shredder efficiency for litter decomposition.	12 µg/L pulse of imidacloprid for 12 hours. 250 mL cages with a stock of 10 adult gammarids together.	Imidacloprid presence	Leaf shredding efficiency / leaf shredding activity; population	Population followed logistic growth function, carrying capacity of 200 for alder. No effect of imidacloprid pulses on population levels and litter decomposition detected. Number of brood carrying females lower in treatments vs. control groups.
6	Conducted acute toxicology tests with *Daphnia magna, Americamysis bathia, Chironomous riparius*, and *Gammarus pulex*. Used a literature search of industry data to conduct this test rather than direct laboratory studies. Selected laboratory EC50 and LC50 tests with a duration of 48-96 hrs.	Literature review and analysis, not applicable	Imidacloprid presence; species	Mortality	Crustaceans, especially D. magna, are "substantially less sensitive than aquatic insects." They recommended that 48-h acute tests on *Chironomus* be used as a benchmark for acute tests, and 28-day water-spiked *C. riparius* test be used for longer-term exposure.
7	Looked at relationship	Dissolved imidacloprid	Imidacloprid	Immobility	96 h EC50 (immobility) for *I.*

	between temperature and time-to-effect for imidacloprid with mayfly *Isonychia bicolor*. Used acute toxicity tests using sublethal endpoints and mortality, respirometry, and radiotracery assays. Each experiment was run "in triplicate", with groups of 6 larvae. Temperature was 15° C (+/- 0.11) with a 12:12 photoperiod.	(SigmaAldrich, purity 99.9%) in soft water with 0.1% DMSO carrier. Concentration (4.4 mg/L) determined using imidacloprid ELISA. Exposure concentrations for imidacloprid were 1,2,4,8,10,20,40, 80, 100 µg/L. Did not feed animals.	presence; temperature	and mortality	*bicolor* at 15° C was 5.81 µg/L, which is 3.2 fold lower than 50% mortality. Uptake experiments with 14° C imidacloprid found initial uptake rates increased with increasing temperature. Separate experiments with other aquatic insects found that all of them uptook more imidacloprid with increasing temperatures. "[P]rofound impairments of motor function were evident far before mortality" (49).
8	Compared chronic toxicity of imidacloprid and two other chemicals on *Chrionomus dilutus*. Grew *C. dilutus* in lab at 23+/- 1.0 ° C with 16:8 photoperiod. Adults given 3 days to produce eggs. To avoid disturbance, isolated jars, checked for egg masses daily.	Larvae exposed to 0, 0.1, and 0.3 µg/L of imidacloprid. On day 14, removed all treatments, counted and measured organisms, and maintained them for 26 days to take notes on emergence.	Imidacloprid and two other chemicals' presence	Mortality, weight (wet)	14-day L50 for imidacloprid was 1.52 µg/L. 40-day EC50 on emergence was 0.39 µg/L for imidacloprid (vs. an average of 4.13 µg/L).
9	**Tested imidacloprid affect on symmetry of damselfly *Coenagriidae* body parts. Collected damselfly larvae**	**Dissolved 15% monosultap triazophos EC (Suke Agriculture and Chemistry Co. Ltd, Jiangsu Province,**	**Imidacloprid presence**	**Mortality, participants' left and right sides,**	**No significant correlation between size and difference between sides.**

	from wild. Fed *ad libertum* at 20°C, 12:12 photoperiod. Used 30 larvae with mean head-width of 2.59 mm. Rearing medium replaced and aerated every 48 hours. Reared for 20 days.	**China), in tap water. The final insecticide concentrations were: 1.50 ng/L, 15.0 ng/L, and 150 ng/L.**		**body length, first femur length, first tibia length, second femur length, second tibia length, third femur length, third tibia length.**	**Found significant effects of insecticide on symmetry for femur and tibia.**
10	**Determined effects of imidacloprid and fipronil (separately) on *Sympetrum infuscatum* (*Libellulidae*) larvae and adults in rice patties after a one-time application. Experiments were outside, but isolated from other elements. Experiment lasted 3 months (waited for emergence).**	**Maximum of 52.8 µg/L at one day and 1.3 µg/L at 6 hour of imidacloprid and fipronil per day. Applied as commercial granular formulations of Admire Box Granule (2% imidacrlopid, Bayer Cropscience K.K., Japan) to nursery boxes containing 32-day old rice seedlings at a rate of 50 g of granules per nursery box as recommended.**	**Imidacloprid presence**	**Mortality, emergence (successful or no)**	**Both pesticides dissipated quickly -- 8.8 day half life for imidacloprid. Larvae and exuviae in the fipronil-treated rice patty decreased 63.6% +/- 18.2%, 15.2% +/-6.0% , and 0% in the fipronil, imidacloprid, and control patties. Imidaclopird significantly reduced successful emergences.**
11	Field study of how imidacloprid applications affected populations of	10 kg/ha application of imidacloprid. Collected water samples (2L) after 2	Imidacloprid and dinotefuran	Population of different species	Maximum concentrations of imidacloprid in water was 157.50 µg/L at

	aquatic insects. Measured abundance of ***Crocothermis servilia mariannae****, Lyriothermis pachygastra,* ***Orthetrum albistylum speciosum,*** *Notonecta triguttata*, and *Guignotus japonicus.* Set up six separate rice mesocosms in water tanks with soil sediments containing seed banks. To avoid photolysis and degradation, samples were collected in amber bottles sealed with aluminum foils and stored in a freezer at -30°C.	hours on the first day, then 1, 3, 14, 28, 56, 91, and 119 days after transplanting using random sampling spots.	presence		two-hour mark, but soon decreased to below 0.10 µg/L in three days. Imidacloprid half-life was < 3 days. Maximum imidacloprid concentration in soil was 13.55 µg/kg at 14 days after transplanting, residue of 1.50 µg/L throughout experiment. For predators, predators like *Guignotus japonicus* and *Crocothemis servilia* nymph populations dropped after imidacloprid treatment, while *N. triguttata* and *Lyriothemis pachygastra* nymphs were at first high in imidacloprid treatments but then declined or disappeared over time.
12	Exposed first instar larvae of *Chironomus riparius* to a mixture of imidacloprid, thiacloprid, deltamethrin, and esfenvalerate in the same mix over 96 hours in a 1-hr pulse. Then reared the larvae in a clean area for rest of their life.	0.068 µg/L for deltamethrin, 0.13 µg/L for esfenvalerate, 5.4 µg/L for imidacloprid, and 4.6 µg/L for thiacloprid	Mixture application of imidacloprid, thiacloprid, deltamethrin, and esfenvalerate	Survival, development time, fecundity	Increased mortality of *C. riparius* for most pesticide exposures. ANOVA found that development time was about the same. No difference in fecundity.
13	Exposed *Chironomus dilutus*	Used Admire 240F for	Imidacloprid,	Mortality.	96-h LC50 for imidacloprid was

	larvae to a mixture of chlorpyrifos, imidacloprid, and dimethoate in addition to the three chemicals separately. Used 96-hour static bioassays with single, binary, and ternary mixes. Raised the larvae from eggs, tested on 10-day old larvae at 23°C with 16:8 hour light to dark schedule.	imidacloprid from Bayer CropScience. Stock solutions stored at 4 C in amber glass bottles. Determined LC50 values of each chemical individually, then for final study added that amount in a 1:1 ratio to water. Tried 5 different concentrations; imidacloprid was tested at 0.842, 1.39, 11.2, and 22.2 µg/L.	chlorpyrifos, and dimethoate presence, concentration, and mixture	Checked DO, temperature, pH, ammonia, and conductivity at 0 and 96 hours (so at the start and the end).	2.65 µg/L (vs. 0.634 µg/L for chlorpyrifos). Overall, Dimethoate was much less toxic than chlorpyrifos or imidacloprid. Chlorpyrifos and dimethoate were not additive. Chlorpyrifos reacted synergistically with imidacloprid and antagonistically with dimethoate.
14	Studied oxidative stress responses and behavior changes in crustacean amphipod *Gammarus fossarum* and the growth rate in freshwater algae *Desmodesmus subspicatus* after 96-hour exposure to imidacloprid.	102.2 µg/L of Confidor 200SL imidacloprid (the commercial form of imidacloprid)	Imidacloprid and 6-chloronicotinic acid presence	Lipid peroxidation, catalase activity	Algae growth was very sensitive to 6-chloronicotinic acid and less sensitive to imidacloprid. For amphipods, low doses (102.2 µg/L) were enough to induce lipid peroxidation, and Confidor 200SL induced greater catalase activity (511.3 µg/L) and lipid peroxidation (256.6 µg/L). 6-Chloronicotinic acid changed antioxidant mechanisms / catalase activity but did not change lipid peroxidation levels. More details cannot be found because only the abstract is available.

15	Exposed arthropod *Gammarus pulex* to imidacloprid in 14-day and 21-day tests. Also conducted a starvation experiment without exposure to imidacloprid to show that starvation alone does not explain mortality in constant imidacloprid exposure. Used multiple stressor toxicokinetic-toxicodynamic modeling approach.	Two treatments with two high, one-day pulses of imidacloprid (140 µg/L = 0.59 u µmol/L) and one treatment with low, constant concentration of 15 µg/L.	Imidacloprid presence; concentration	Feeding activity, lipid content, immobility, survival	Feeding and lipid content significantly reduced under exposure to low, constant imidacloprid concentration (15 µg/L). Organisms unable to move and feed. Caused high mortality after 14 days constant exposure. Repeated imidacloprid pulses did not affect feeding and lipid content: *G.pulex* become immobilized during pulse but recovered quickly after transfer to clean water.
16	Established baseline toxicity data for black fly larvae *Simulium vittatum* exposed to imidacloprid and fipronil Used an acute 48-hour orbital shaker toxicity test. Reared black fly larvae and used fifth instar larvae for all of the tests. pH of about 7-8. Used constant temperature, pH, DO, conductivity, alkalinity, and hardness for each test.	Analytical-grade standards of imidacloprid and fipronil from Chemservice, > 98% pure. Dissolved standards in 100 ml of acetone, stored at 4 C. Tested six insecticide concentrations: 2.00, 4.00, 6.00, 8.00, 10.00, and 12.00 µg/L.	Imidacloprid concentration	48-h LC50	Median LC50 for fipronil was 0.19 - 0.29 µg/L; LC50 for imidacloprid was 6.75 µg/L - 9.54 µg/L. Also noted impaired movement in abdominal and thoracic segments, inability to attach to the pan. "[A]ll larvae appeared normal after exposure to imidacloprid at approximately 2 µg/L"
17	Found toxicity of imidacloprid for *Chironomus tentans* and *Hyalella azteca*. Used 96-hour test to find	99.2% pure Admire formula Nominal concentrations of 1,5,29, 145, and 725 µg/L	Imidacloprid presence	Mortality, emergence, species	Lowest-observed-effect concentration (LOEC) for 28-day test on *C. tentans* was 3.57 µg/L. Larval growth became

	acute exposure 96-h lethal concentration. Pulse lasted for four days, and then transferred animals to clean water for 24 days. Assessed on day 10 and day 28.	for *C. tentans* Concentrations of 2,11, 55, 275, 1,375 µg/L for *H. Azteca*			reduced. LC50 and LC25 were 0.91 µg/L and 0.59 µg/L respectively. LC25 for pulse treatment was 3.03 µg/L for *C. tentans.* For *H. Azteca,* tests on day 10-28 constant exposure survival value was 11.95 µg/L, 28 day exposure survival was 11.46 µg/L. *C. tentan*s more sensitive to acute and chronic imidacloprid exposure, but less sensitive to single pulse of *H. Azteca*.
18	Studied sublethal effects of imidacloprid, azadirachtin, deltamethrin, and spinosad to *Aedes aegypti* 4th instar larvae over 10-day trial.	Imidacloprid came from Evidence WG, 700 g a.i./L, from Bayer CropScience. Tested at 0.0, 1.5, 1.5, 3,0, 6.0, and 15.0 ppm for imidacloprid.	Imidacloprid concentration	Mortality, distance swam, resting time, time spent in slow swimming	Five larvae displayed cell death of muscular and nervous cells. Imidacloprid increased pupae swimming distance. Larvae did not exhibit wriggling swimming pattern when exposed to deltamethrin, imidacloprid, and spinosad.

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Table 2: Review of Imidacloprid Levels for Order: Odonata

#	Description	Species	Level	Results
1	Imidacloprid effect on symmetry	Coenagriidae	1.50 nanograms/L (0.0015 µg/L), 15.0 ng/L (0.015 µg/L), 150 ng/L (0.15 µg/L)	Found potential effects of insecticide on symmetry for femur and tibia. Mortality was higher for all imidacloprid trials, yet between the concentrations there were no significant differences among mortality.
2	Effects of imidacloprid and fipronil (separately) on in rice patties after a one-time application.	*Sympetrum infuscatum* (Libellulidae) larvae and adults	Maximum of 52.8 µg/L of imidacloprid at one day after treatment. Imidacloprid concentration decreased to 13.2 µg/L by 14 days after transplantation and 4.9 µg/L after 30 days.	Larvae and exuviae in the fipronil-treated rice patty decreased 15.2% +/-6.0%. Imidaclopird significantly reduced successful emergences by about 90%.
3	Field study of how imidacloprid applications affected populations of aquatic insects.	*Crocothermis servilia mariannae, Orthetrum albistylum speciosum,*	10 kg/ha application of imidacloprid. Maximum concentrations of imidacloprid in water was 157.50 µg/L at two-hour mark, but soon decreased to below 0.10 µg/L in three days.	Nymph populations dropped significantly (over 50%) after imidacloprid treatment,

4	Literature review of imidacloprid effects on 12 orders of aquatic insects, including Odonata	Odonata -- various	Not applicable – literature review	Geometric mean (µg/L) of LC50 for 24-94 hour test for Odonata was 55.2 µg/L; final recommendation for aquatic insects as a whole was to keep chronic exposure below 0.2 µg/L.

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